# Alleviative Effect of Isorhamnetin and Its Derivatives on Nonalcoholic Steatohepatitis

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# Abstract

Nonalcoholic steatohepatitis (NASH) is the most severe and progressive form of nonalcoholic fatty liver disease which affects people who do not or little drink alcohol. The pathogenesis is mostly explained by 'Two-hit hypothesis' representing intra-hepatic lipid accumulation due to metabolic syndromes as the 'first hit' and followed by the 'second hit' resulting in increased inflammation and liver injury with fibrosis. A build-up of fat in the liver so-called steatosis is usually considered benign, but it may progress to more severe pathologic condition over the course of several years when it is combined with inflammation, injury, and fibrosis resulting in NASH which can in turn potentially progress to end-stage liver diseases such as cirrhosis and hepatocellular carcinoma. The fact that NASH pathologic features are reversible which are manifested just before irreversible end-stage liver diseases, it emphasizes the importance and urgency of finding a treatment of NASH to prevent patients to progress into advanced disease steps. However, so far, there are still no drugs approved to treat NASH.

On the other hand, flavonoids are polyphenols widely presented in fruits, vegetables, stems, seeds, etc. Flavonoids are shown to have bioactive effects against metabolic diseases in addition to their strong antioxidant activities. Quercetin is widely studied flavonoid with potentials against carcinoma, inflammation, fibrogenesis, and oxidative stress. Isorhamnetin is an immediate metabolite of quercetin, also found in various plant extracts and plant-derived products. It has been shown to prevent oxidative stress in HepG2 cells [1], inhibit lung and breast cancer cell proliferation [2,3], improve inflammatory bowel disease [4], and repress adipogenesis in 3T3-L1 cells [5]. Research studies in rodents including investigations in our laboratory, reported the antiobesity, antioxidant, and antifibrotic effects of isorhamnetin or plant extracts rich in isorhamnetin [6,7]. Interestingly, bioavailability reports showed that the most part of absorbed quercetin is found in its methylated form – isorhamnetin which is maintained in plasma longer than quercetin. It strongly implies a potential role of isorhamnetin as a main mediator of beneficial effect of quercetin. Structurally, previous comparative analysis revealed that aglycone flavonoids exert more biological activity compared to their glycones.

Thus, firstly we investigated the effect of isorhamnetin on NASH pathologic features in the liver. NASH was induced in C57BL/6 mice and treated with isorhamnetin orally. Liver and serum were isolated from experimental groups for biometrical, biochemical, histological, gene expression, and micro array analysis. Great number of genes mainly involved in lipid metabolism, oxidation reduction process, and fatty acid metabolism were altered following the induction of NASH. The number of altered genes were remarkably decreased by the isorhamnetin treatment. Consistently, genes involved in fatty acid metabolism, steroid biosynthesis, and PPAR signaling pathway were invariably decreased. In addition, isorhamnetin treatment reduced intrahepatic lipid accumulation associated with lower triglycerides content and inhibited *de novo* lipogenic pathway in NASH-induced mice. Liver injury markers in serum were consistently improved when treated with isorhamnetin compared with their non-treated NASH-induced littermates. Along with the anti-steatosis effect, isorhamnetin reduced fibrogenic marker gene expressions accompanied with the reduced area of collagen deposition on liver sections. In addition, number of apoptotic cells were significantly decreased after the treatment. Analysis in adipose tissue revealed the infiltration of macrophages in NASH-induced mice showing a chronic inflammatory state while the treatment with isorhamnetin alleviated this inflammatory condition in adipose tissue.

We next sought to identify the structure-activity relationship of methyl group by which isorhamnetin differs from quercetin on the development of fibrosis. To test this hypothesis, we synthesized five different mono-methylated derivatives of quercetin namely isorhamnetin, azaleatin, 3-methylquercetin, tamarixetin, rhamnetin. Hepatic fibrosis is initiated primarily by the hepatic stellate cells (HSC). Chronic injury to liver caused by metabolic disorders, alcoholism, viral infections, and NASH can lead to transdifferentiation of HSCs from its quiescent resting state into its activated state characterized by more migratory, proliferative, and contractile myofibroblast-like phenotype. The activated HSCs promotes extracellular matrix (ECM) molecules including different types of collagens leading to development of fibrosis, and further hepatic injuries which are irreversible. Thus, we used HSCs as an *in vitro* fibrosis model. Fibrosis was induced by transforming growth

factor-β (TGFβ) in rat stellate cells (HSC-T6), and then cells were treated with methylated derivatives at various dose and time. Immunofluorescence staining of collagen type I (Col1) and alpha smooth muscle actin (αSMA), cell proliferation assay, and fibrogenic gene expression analysis were conducted. All derivatives showed antiproliferative effects in dose- and time-dependent manner in HSC-T6 cells. Next, TGFβ-induced stellate cell was treated with 20 μM and 40 μM of derivatives for 24 hours and isorhamnetin, 3MQ, and RHA reduced the protein level and mRNA expression of *Acta2* (gene encoding αSMA); 3MQ prevented the augmentation of *Col1a1* (gene encoding Col1) in TGFβ-induced stellate cells. Each compound had different effects against pathologic features of fibrosis which suggests that hydroxyl position plays an important role in the regulation of anti-fibrotic activity of compound. However, the molecular mechanism underlying their antifibrotic effect remains to be elucidated. Our data demonstrated for the first time that methylation could improve the antifibrotic effect of quercetin.

In conclusion, these findings collectively suggest that isorhamnetin elicits beneficial effect on hallmarks of NASH by improving steatosis, injury, and fibrosis in a novel human like NASH-induced mice. This hepatoprotective effect of isorhamnetin was correlated to the inhibition of *de novo* lipogenic and fibrogenic gene expressions; alleviation of liver triglycerides (TG) content; and diminution of hepatic collagen deposition accompanied with the reduced number of apoptotic hepatocytes. Thus, isorhamnetin can be a novel candidate for the additional compound in NASH drug development. Moreover, the addition of methyl group on functionally important position may enhance the antifibrotic effect of quercetin. Further evidence with human NASH will be required to understand the effect of isorhamnetin on NASH development.

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